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Effects of ethanolic extract of *Raphia hookeri* fruits on chilled ram semen characteristics

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ABSTRACT

Oxidative stress which result from the imbalance between reactive oxygen species (ROS) and the capacity of antioxidants to neutralize free radicals, contributes to the deterioration of semen quality during preservation. *Raphia hookeri* fruits are rich in compounds with antioxidants properties such as phenols, flavonoids, tannins, saponins, anthocyanin and coumarin. The present study aims at evaluating the effect of the supplementing semen extender (skim milk) with different levels of ethanolic extract of *Raphia hookeri* fruits on chilled ram semen stored. Semen samples from six healthy adult Djallonké (*Ovis aries*) rams were collected once a week for 6 weeks using electro-ejaculator and diluted in skim milk-based extenders. This mixture was supplemented with *Raphia hookeri* 75, 150, and 300µg/ml ethanolic extract at doses added to extender, alongside neutral control and positive control (0 µg/ml of extract, 3000 µg/ml of vitamin C). Samples were therefore preserved at 5 °C in the fridge. Sperm cell characteristics were assessed consecutively after 0, 24, 48, and 72 hour, using CASA for the kinematics characteristics, eosin/nigrosin staining for the viability, hypo-osmotic swelling test for membrane integrity and that of DNA was assessed through the orange acridine test. The supplementation of the skim milk extender with various doses of Raphia hookeri extract provided higher percentages of viability and percentages of motile sperm cells at 72 hour of storage. Moreover, 150µg/mL extract added to the extender permit to maintain the DNA integrity at 100% over the time of preservation and the membrane integrity higher than the negative control at 72 hour. Concerning the oxidative stress biomarkers, 300µg/mL permit to reduce the accumulation of MDA at 72 hour and maintain the Catalase (CAT) and Superoxide Dismutase (SOD) close to the negative control value. However, no significant effects of the interaction of the duration of storage and the dose of *Raphia hookeri* have been noted during this investigation. Further research has to be performing to determine the various doses with significant effects. *Raphia hookeri* extract showed promise as a natural antioxidant, with trends indicating potential protective effects against oxidative damages on ram spermatozoa.

Key words: Liquid storage, Oxidative stress, Raphia hookeri, Ram, Semen cells

INTRODUCTION

Sheep farming is a sector of paramount importance in many countries worldwide (Reddy, 2020). This species was among the first to be domesticated in Southwest Asia, approximately 11,000 years ago and is now raised across the globe (Kawęcka *et al.*, 2022). Sheep farming serves as a vital source of income for many families living in resource-limited areas (Odintsov Vaintrub *et al.*, 2021). According to 2018 FAO data, the global sheep population stood at 1,173 million head, distributed as follows: 43.6% in Asia, 30% in Africa, 11.2% in Europe, 8.1% in Oceania, and 7.1% in the Americas. In Cameroon, sheep meat production increased slightly from 25.1 thousand tonnes in 2022 to 25.6 thousand tonnes in 2023 (MINEPIA, 2024).

Among livestock species, sheep plays a strategic role due to its high fertility rate, short lambing interval, multiple offspring, adaptability in arid areas, and capacity to transform poor vegetal materials to high meet and milk value product. However, the sheep sector faces multiple constraints like unavailability of males with great phenotypic characteristics and transportation issues. To increase productivity, intensifying reproduction through artificial insemination (AI) is a viable alternative. It allows for the rapid dissemination of elite genetic material without the need for a large number of breeding males. Nevertheless, semen preservation for the AI faces obstacles. The two most significant causes are thermal shock (Hammerstedt *et al.,* 1990) and oxidative stress (Bucak *et al.,* 2010) which lead to the deterioration of semen quality. Indeed, oxidative stress results primarily from an imbalance between reactive oxygen species (ROS) and the capacity of antioxidants to neutralize free radicals.

Naturally, seminal plasma contains both non-enzymatic and enzymatic antioxidants. However, dilution for preservation purposes considerably reduces its capacity to neutralize oxidative stress (Peris-Frau *et al.,* 2020). Hence, the type of diluent is a critical factor in slowing metabolism and limiting reactive oxygen species (ROS) production. The generation of ROS impairs sperm kinematics, viability, morphology, the integrity of the plasma membrane and DNA (Barati *et al.*, 2020). These damages significantly reduce post-storage fertility and limit the effectiveness of AI (Ritchie and Ko, 2021). In this light, the incorporation of antioxidants into semen diluents represents a promising approach. To address these issues, a wide range of cryoprotectants (Morris *et al.*, 2007) and synthetic antioxidants have been used in the preservation of ram semen. Specimens include keratin, cysteamine, catalase, and cysteine. But, these substances are very expensive and inaccessible in certain regions. Given the scarcity of these antioxidants and the aim of significantly reducing the cost of artificial insemination, several studies such as that of El-Harairy *et al.* (2018), have been conducted to identify plant extracts capable of performing the same antioxidant functions. Extracts from medicinal plants, rich in polyphenolic compounds, could limit oxidative damage and maintain the quality of stored sheep semen. The *Raphia hookeri* plant is widely used in human nutrition and medicine due to its antioxidant effect as well as in the prevention of cardiovascular diseases in human health (Ogbuagu, 2008). The mesocarp of the *Raphia hookeri* fruit is particularly rich in phenolic compounds, tannins, flavonoids, alkaloids, and saponins, which confer antioxidant properties (Okwu *et al.*, 2008). Due to the accessibility of this plant and it phytochemical status, it could be used as a natural source of antioxidants and improved the ram liquid preservation of the semen.

The overall objective of this study is to assess the effects of ethanolic extract of *Raphia hookeri* fruit, a plant with antioxidant activity, on the chilled ram semen stored for 72 hours in a skim milk base diluent, with a view to improve the storage capacity. More specifically, to evaluate the effects of semen supplemented with *Raphia hookeri* 75, 150, and 300µg/ml ethanolic extract at doses added to extender on the motility, viability, normality, plasma membrane integrity, oxidative stress markers and kinematic parameters of ram spermatozoa.

MATERIALS AND METHODS

Study site and period

The present study was conducted at the Teaching and Research Farm and the Laboratory of Health Physiology and Animal, Department of Animal Sciences, Faculty of

Agronomy and Agricultural Sciences, University of Dschang. The study was carried out from April to July 2026.

Experimental animals and feeding

Six (6) healthy Djallonké rams, aged 2.5 - 3 years, weighing 40 ± 2 kg were used for the present study. They were housed at the Teaching and Research Farm of the University of Dschang (situated between latitudes 5°44′–5°36′ N and longitudes 10°06′–9°94′ E with a mean annual temperature of 20°C). Animals had free access to good quality of forage and water.

Ethical considerations

The experimental protocol used in this study was approved by the Department of Animal Science of the Faculty of Agronomy and Agricultural Sciences of the University of Dschang, Cameroon and in strict compliance with guidelines for care and manipulation of animals.

Extract preparation

Raphia hookeri fruits were harvested from the same tree in Dschang and oven-dried at 40°C for six days. The dried fruits were then milled into a fine powder using a blender. For ethanolic extraction, 100 g of the powdered material was macerated in 1000 mL of 95% ethanol at room temperature for 48 h. The mixture was filtered through Whatman No. 1 filter paper, and the ethanolic filtrate was oven-dried at 45°C until a constant weight was obtained. The extraction yield was 12.8%, and the resulting extract was stored in a freezer until further use. The extraction yield was calculated using the following formula:

Yield=(Weight of extract/Weight of powder)×100

Determination of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH assay for the samples was performed as described by Mensor *et al.* (2001). In a 96-well plate, 20 µL of methanol was added to the wells. Then, 20 µL of the methanolic sample solutions (2 mg/mL) was introduced into the first two wells of each column (four columns were used per sample), and serial twofold dilutions were performed in the subsequent wells, maintaining a volume of 20 µL. A volume of 180 µL of methanolic DPPH solution (0.08 mg/mL) was added to each well of the first three columns, while 180 µL of methanol was added to each well of the fourth column. The plates, containing a final volume of 200 µL per well, were incubated for 30 minutes in the darkness at room temperature. After incubation, optical densities were measured using a spectrophotometer (FLUOstar Omega microplate reader) at 517 nm and converted into percentages of antioxidant activity. Vitamin C (L-ascorbic acid) was used as a positive control. Three replicates were performed for each sample. The percentage of antioxidant activity for each sample was calculated using the following formula:

Assay = Sample + Methanolic DPPH solution
Blank = Sample + Methanol

The various DPPH inhibition percentages were used to determine the concentrations required to inhibit 50% of the DPPH radical (IC50) (Yassa *et al.*, 2008). To do this, regression lines were plotted using the various antioxidant activity percentages and the base-10 logarithm of the sample concentrations that means, [% = f(logC)]. Regression equations of the form y = ax + b were used. IC50 values were calculated for each extract by setting y = 50, resulting in the relationship IC50 = (50 - b)/a.

% antioxidant8 activity=[Absorbance of DPPH-(Absorbance of essay- Absorbance of blank)]×100

Extenders preparation

Skim milk extender used for the study was prepared as follow. 22 g of skim milk powder, 100 IU/ml of penicillin and 0.5 mg/ml of streptomycin were weighed using a sensitive balance with a capacity of 300 g and accuracy of 10^{-3}, then introduced into a 200 ml volumetric flask 50ml of distilled water, topped up with distilled water up to the 200ml mark, homogenized with a magnetic stirrer. This solution of pH 7±0.2 and an osmotic pressure of 295 mOsmol/kg was heated to 95°C for 10 minutes, filtered through Whatman filter paper (N°1) and stored at 5 °C until use.

The extender thus prepared was used to prepare the 5 extenders tested during the trial, by adding additives:

1. Negative control 0 (0vitamin C),
2. Positive control 0+ (3000 µg/ml of vitamin C),
3. LT75 (ethanolic extract of *Raphia hookeri* added at dose 75 µg/ml),
4. LT150 (ethanolic extract of *Raphia hookeri* added at dose 150 µg/ml),
5. LT300 (ethanolic extract of *Raphia hookeri* added at dose 300 µg/ml).

Semen collection

Semen collection was performed by means of Electro ejaculation (EE) following the method described by Marco-Jiménez *et al.* (2005), with minor modifications (neither sedative nor anaesthetics was given). Electrojac V® stimulator (Ideal instruments, Neogen Company, Lansing, MI, USA) was used. After assessing the macroscopic (viscosity, color) quality of the semen and the sperm motility, high-quality semen samples were selected and mixed. The dilution was assessed by adding in a 20µl micro tube, 19µl of each extender and 1µl of the fresh. The samples were then supplemented at various levels of *Raphia hookeri* extract and stored in the refrigerator at 5 °C.

Sperm evaluation

The analyses were performed at consecutive various time points: 0, 24, 48, and 72 hour after the preparation of the samples. After homogenization and 10 minute incubation at 37°C, a 10 µL aliquot of each sample was placed in a chamber of the CASA cell. The analysis was performed using the Sperm Class Analyzer® v.6 system (Microptic), equipped with a microscope at 100x magnification. For each sample, 5 fields were analyzed, as recommended by Broekhuijse *et al.* (2012). The measured parameters were: density, x10^6/mL; the percentage of motile spermatozoa, the percentage of spermatozoa with progressive motility, rapid progressive motility, and local motility, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), in µm/s, amplitude of lateral head movement (ALH), in µm, and cross-beat frequency (BCF), in Hz.

Assessment of ram sperm viability and morphology

To assess sperm viability, one (1) drop of semen preheated to 37 °C was added to three (3) drops of the eosin/nigrosine mixture preheated to 37 °C. After 2 minutes of incubation at room temperature, this mixture was placed on a microscope slide. A thin smear was then prepared to distinguish between dead spermatozoa, which were stained pink and live spermatozoa, which remained unstained upon observation under a microscope at 400 x magnifications (Fig. 1). Out of a total of 200 spermatozoa counted, the percentage of live and dead spermatozoa was determined for each slide. The slides used to assess sperm viability (stained with a mixture of eosin and nigrosine) were also used to evaluate sperm morphology according to (WHO, 2021) criteria. Abnormalities mainly involved abnormalities of the heads, detached heads, mid-pieces, and tails (Fig. 2). For each slide, the percentage of normal and abnormal spermatozoa was determined, based on a total of 200 counted spermatozoa.

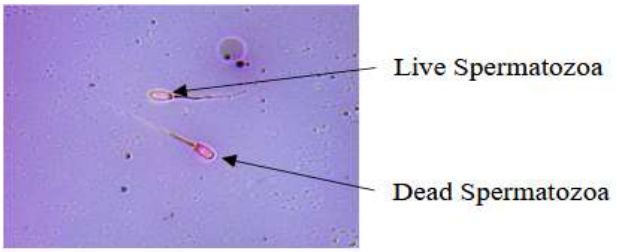

Fig. 1. Assessment of sperm viability (eosin/nigrosine test), observation under a light microscope at 400x magnification

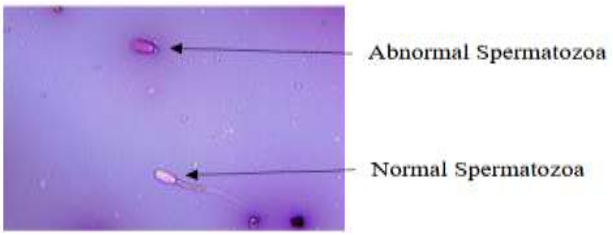

Fig. 2. Assessment of sperm morphology (eosin/nigrosine test), observation under a light microscope at 400x magnification

Assessment of plasma membrane integrity in ram spermatozoa

To assess sperm membrane integrity, 100 µL of semen preheated to 37°C was added to 1,000 µL of a hypo-osmotic fructose solution (100 mOsmol/L) at 37°C.

Fig. 3. Assessment of sperm plasma membrane integrity (fructose hypo-osmotic test), observation under a light microscope at 400x magnification

After 60 minutes of incubation at room temperature, this mixture was then placed on a microscope slide. A thick smear was prepared, dried on a heating stage at 37°C, and observed under an optical microscope at 400 x magnifications (Fig. 3) as indicated by England and Plummer (1993). Spermatozoa with intact plasma membranes at the time of the test absorb the solution and exhibit coiled tails, whereas those with disintegrated plasma membranes have uncoiled tails. Out of a total of 200 counted spermatozoa, the percentages of spermatozoa with intact plasma membranes and those with disrupted plasma membranes were determined for each slide.

Assessment of DNA integrity in ram sperm

The protocol by Tejada *et al.* (1984) was followed. Orange acridine stains the DNA of sperm with intact (double-stranded) DNA green and that of sperm with denatured DNA red (Silva and Gadella, 2006). To this end, a thick smear was prepared on a microscope slide, dried on a heating plate at 37 °C, and fixed in Carnoy's solution (methanol/acetic acid 3:1) for 2 hours.

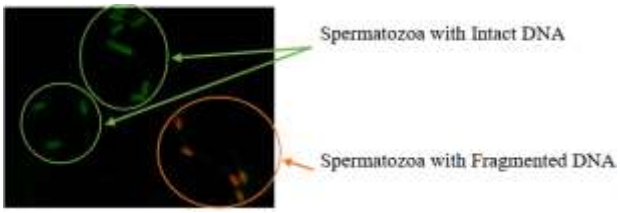

Fig. 4. Assessment of sperm DNA integrity (acridine orange test), observation under a light microscope at 400x magnification

Staining was performed by immersing the slides in an orange acridine solution (0.9 mg/mL, pH 2.5) for 5 minutes. After rinsing, the slides were dried and sealed with resin. Observation was conducted using a fluorescence microscope at 400x magnification (Fig. 4). Out of a total of 200 counted spermatozoa, the percentages of spermatozoa with intact DNA and those with fragmented DNA were determined for each slide.

Antioxidant effects of *Raphia hookeri*

At the different incubation times (6, 24, 48 and 72 h), semen samples were centrifuged at 550 g for 10 min and the pellet was discarded. The supernatant was again centrifuged at 550 g for 10 min and finally at 3000 g for 30 min. The resulting supernatant, considered as the medium, was used to evaluate semen oxidative status.

Evaluation of lipid peroxidation

Lipid peroxidation in the extended semen samples was assessed by measuring malondialdehyde (MDA) levels in the medium. Briefly, 500 µL of 1% orthophosphoric acid solution and 500 µL of a precipitating mixture containing 1% thiobarbituric acid and 1% acetic acid were mixed with 100 µL of each sample in glass tubes. The tubes were incubated at 100°C for 15 min, cooled, and centrifuged at 1000 × g for 10 min. The absorbance of the supernatant was then measured against a blank at 532 nm using a spectrophotometer. MDA concentration was calculated using the Beer–Lambert equation and normalized to the protein content.

$$\textit{MDA concentration} = \textit{Optical desnsity at } 532\ nm \varepsilon \times L \times PC$$

ε : Molar extinction coefficient $1.56 \times 10^5\ M^{-1}cm^{-1}$

L: Light path (cm)

PC: Protein content ($g.L^{-1}$)

MDA concentration ($\mu M.\ g^{-1}$ of protein).

Evaluation of superoxide dismutase activity

An aliquot of medium (140 µL) was mixed with freshly prepared carbonate buffer (1660 µL, pH = 10) and adrenalin (200 µL, 0.3 M). The absorbance was read against the blank at 480 nm after 0.5 and 1.5 min of the initiation of the reaction. The inhibition percentage (I) and the SOD, expressed in units per mg of protein (U/mg), was calculated as indicated below, one-unit total SOD activity was considered as the amount of protein causing 50% inhibition of adrenalin auto-oxidation.

$$I = 100 - (\Delta OD sample \Delta OD blank) \times 100$$

$$SOD\ activity = (I50 \times protein\ concentration)$$

OD: Optical density

ΔOD sample: Difference between OD of the sample read at 1.5 min and OD of the sample read at 0.5 min

ΔOD blank: Average of the differences between OD of the blank read at 1.5 min and OD of the blank read at 0.5 min

I: Inhibition percentage.

Evaluation of catalase activity

To assess CAT activity, an aliquot (50 µL) of the medium of each sample was added to phosphate buffer (500 µL) in glass tubes. Subsequently, hydrogen peroxide (200 µL, 0.2 M) was added to each tube. After one-minute incubation at room temperature, one milliliter of the mixture dichromate-acetic acid (5 % dichromate: glacial acetic acid; 1:3 V/V) was added to the preparation to stop the reaction. The entire preparation was then homogenized and incubated in water bath at 100°C for 10 min and cooled. Finally, the optical density of the content of each tube was read at 570 nm using a spectrophotometer. The concentration of hydrogen peroxide in the sample was determined using a standard curve developed for this purpose.

Evaluation of total peroxidase activity

For this purpose, 0.5 ml of the medium of each sample was added to 1 ml potassium iodide (KI) solution (10 mM) and 1 ml sodium acetate (40 mM) was added. The absorbance of potassium iodide was read at 353 nm, indicating the amount of peroxidase. Then 20 µl of H_2O_2 (15 mM) was added and the change in absorbance over 5 min was recorded. Units of peroxidase activity were expressed as the amount of enzyme required to change optical density by one unit per minute. The enzymatic activity of peroxidase was deduced by Beer-Lambert's law as follows:

OD = Ɛ.C.L

OD: Optical density;

Ɛ: Molar extinction coefficient of peroxidases (11.3 $M^{-1}cm^{-1}$);

C: Total peroxidase concentration;

L: Optical path length (1 cm).

Statistical analyses

Statistical analyses were performed using SPSS v26.0 software. The effects of different doses of extract and the duration of preservation on the chilled of ram semen characteristics were tested using a two way analysis of variance (ANOVA). Student's test was used to separate means when significant differences were found. The relationships between the various sperm characteristics were established through Pearson's correlation coefficient. The threshold for statistical significance was set at 5%. The results have been expressed as mean ± standard deviation.

RESULTS

Phytochemical analysis of *R. hookeri* extract

This result revealed the presence of phenols, flavonoids, tannins, saponins, anthocyanin and coumarin compounds in the *Raphia hookeri ethanolic* extract (Table 1).

Table 1. Phytochemical compounds of *Raphia hookeri* fruits

Compound	Result
Alkaloids	–
Phenols	+
Flavonoids	+
Terpenoids	–
Tannins	+
Saponins	+
Anthocyanins	+
Anthraquinones	–
Steroids	–
Coumarin	+

Quantitative antioxidant activity of *R. hookeri* fruit extract

The *Raphia hookeri* fruit extract exhibited strong antioxidant activity, although its activity was lower than that of the positive control (vitamin C). The extract showed a higher DPPH IC_{50} value than vitamin C, indicating lower DPPH radical-scavenging activity. In the FRAP assay, the extract also showed a lower reducing capacity than vitamin C. The quantitative antioxidant activities of the extract and vitamin C are presented in Table 2.

Table 2. Quantitative antioxidant activity of *R. hookeri* fruit extract

	DPPH (IC_{50})	FRAP [$FeSO_4^{2-}$]
Extract	49.39± 2.65	125.62±3.49
Vitamin C	5.85±1.02	165.65±2.62

DPPH=2,2-Diphenyl-1-picrylhydrazyl; FRAP= ferric reducing antioxidant power

Effects of ethanolic extract of *R. hookeri* fruits and the preservation period on the percentage of motile spermatozoa and the density

These results indicate no significant effect (p>0.05) of the interaction of time and Raphia hookeri extract on the various parameters. Regarding density, the highest value was recorded at 48 hour of storage at 336.97 ± 60.73, while the lowest was observed at 72 hour at 290.56 ± 57.74 (Table 3). For POMS, a progressive decrease was observed during storage, from 87.08 ± 4.08 at 0 hour to 79.00 ± 8.34 at 72 hour. However, the sample supplemented at 300µg/ml recorded the highest percentages (81.08±6.56%) of motile spermatozoa after 72 hour of preservation.

Table 3. Effects of different doses of ethanolic extract from *R. hookeri* fruits and the preservation period on the density and the percentage of motile spermatozoa

Characteristics	Treatments	Duration of storage				Interaction P-value
		0 H	24H	48H	72H	
Density	Negative control	280.79±64.58	306.02±70.62	295.94±26.33	280.80±64.58	.375
	Positive control	278.28±44.01	275.62±44.16	378.13±40.42	313.44±66.00	
	LT 75	313.84±71.38	294.94±54.04	302.73±61.06	293.55±55.48	
	LT 150	327.11±59.22	318.67±55.28	376.31±49.59	271.49±69.27	
	LT 300	303.99±69.86	293.35±42.42	331.72±77.19	267.01±68.49	
POMS	Negative control	84.73±4.08	84.51±3.56	85.96±6.15	77.86±10.25	.926
	Positive control	86.96±2.76	86.04±5.53	82.08±7.32	77.98±7.70	
	LT75	88.37±2.89	85.79±3.10	83.94±5.27	78.91±9.68	
	LT150	85.35±6.45	84.25±5.83	83.06±4.26	79.14±10.36	
	LT300	89.96±1.58	85.75±3.52	79.40±9.74	81.08±6.56	

Negative control= 0 (ovitamin C), Positive control 0+ (3000 µg/ml of vitamin C); LT75= ethanolic extract of Raphia hookeri added at dose 75 µg/ml; LT150=ethanolic extract of Raphia hookeri added at dose 150 µg/ml; LT300= ethanolic extract of Raphia hookeri added at dose 300 µg/ml ; POMS= Percentage of Motile Spermatozoa

Table 4. Effects of different doses of ethanolic extract from *R. hookeri* fruits and the preservation period on viability, morphology, membrane and dna integrity of spermatozoa

Characteristics	Treatments	Duration of storage				Interaction P-value
		0 H	24H	48H	72H	
Viability	Negative control	93.60±3.20	85.00±6.44	78.80±9.55	70.80±9.57	
	Positive control	94.00±3.31	88.40±2.60	87.40±5.81	82.20±8.58	
	LT75	92.60±1.67	89.40±4.56	87.40±8.05	81.40±6.18	.462
	LT150	96.40±1.51	92.00±3.74	89.40±5.07	82.40±8.53	
	LT300	92.20 ±0.83	89.20±3.34	86.20±2.58	85.20±7.53	
Morphology	Negative control	99.00±1.41	98.00±1.87	97.80±.83	95.60±4.50	
	Positive control	98.00±.70	99.40±.54	98.00±1.22	94.40±6.42	
	LT75	99.60±.89	98.60±1.94	96.00±5.43	95.20±4.86	.974
	LT150	99.00±1.22	97.80±2.77	98.60±.89	94.80±5.49	
	LT300	99.40±.89	98.80±.44	98.00±1.41	96.60±4.27	
Membrane Integrity	Negative control	99.40±1.34	98.60±2.07	99.60±0.54	99.00±1.00	
	Positive control	98.00±2.82	99.40±0.89	98.60±0.54	98.40±0.89	
	LT75	99.00±1.22	98.80±1.30	98.80±1.09	99.60±0.54	.875
	LT150	98.60±1.67	98.20±1.64	99.00±.70	99.60±.89	
	LT300	97.60±4.82	98.40±2.60	99.60±0.54	99.00±0.00	
DNA Integrity	Negative control	100.00±0.00	100.00±0.00	99.60±0.89	98.40±3.05	
	Positive control	100.00±0.00	100.00±0.00	99.40±1.34	99.60±0.89	
	LT75	100.00±0.00	100.00±0.00	100.00±0.00	99.20±1.78	.890
	LT150	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	
	LT300	100.00±0.00	100.00±0.00	100.00±0.00	98.20±4.02	

Negative control= 0 (ovitamin C), Positive control 0+ (3000 µg/ml of vitamin C); LT75= ethanolic extract of Raphia hookeri added at dose 75 µg/ml; LT150=ethanolic extract of Raphia hookeri added at dose 150 µg/ml; LT300= ethanolic extract of Raphia hookeri added at dose 300 µg/ml

Effects of ethanolic extract of *R. hookeri* fruits and the preservation period on viability, morphology, membrane and dna integrity of spermatozoa

The results of the present study showed no significant effect ($p > 0.05$) of the interaction of the time and the level of supplementation with *Raphia hookeri* extract on the various parameters. Viability decreased markedly over time, falling from 93.60 ± 3.20% at 0 hour to 70.80 ± 9.57% at 72 hour for the negative control (Table 4). The 150 µg/mL dose also showed a similar decline, from 96.40 ± 1.51% at 0 hour to 82.40 ± 8.53% at 72 hour. Morphology remained very high in all groups. DNA integrity remained stable up to 48H, averaging 100.00 ± 0.00% in most treatment groups then, decreased slightly to 98.40 ± 3.05% at 72 hour for the neutral control, and to 98.20 ± 4.02% for the 300 µg/mL dose, with no significant difference., the perfect capacity (100%) on the DNA integrity storage has been recorded in samples supplemented at 150 µml of extract over the 72 hour of preservation. From the 0 hour to the 72 hour of preservation. Morphology and membrane integrity showed less variability in all the groups tested. Nonetheless, over the 72 hour of preservation, samples supplemented at 75µg/ml scored the highest (99.60±0.54%) capacity on the membrane integrity preservation whereas the highest morphology preservation (96.60±4.27%) was recorded in diluent supplemented whit 300µg/ml of *Raphia hookeri* extract.

Table 5. Effects of different doses of ethanolic extract of *R. hookeri* fruits and the preservation period on fast progressive (WHO A), slow progressive (WHO B), locale motile (WHO C) and immotile (WHO D)

Characteristics	Treatments	Duration of storage				Interaction P-value
		0 H	24H	48H	72H	
WHO A	Negative control	3.37±0.70	3.10±0.90	4.86±2.05	3.20±2.62	
	Positive control	6.29±3.37	5.07±3.69	2.37±1.51	3.78±2.77	.428
	LT75	4.83±1.85	3.35±1.05	3.95±2.46	3.86±2.19	
	LT150	4.02±1.42	3.61±1.12	3.38±1.510	4.35±2.95	
	LT300	4.97±1.16	3.45±0.27	3.12±1.90	4.34±2.94	
WHO B	Negative control	22.07±4.92	21.87±4.62	26.20±5.63	22.36±11.51	
	Positive control	27.19±5.28	25.73±6.93	18.43±6.26	16.88±9.20	
	LT75	26.55±7.40	21.42±3.56	21.93±8.74	19.97±10.28	.713
	LT150	25.21±8.40	21.90±4.84	22.12±6.21	23.71±10.60	
	LT300	29.83±3.84	23.34±3.97	20.07±8.90	21.79±10.50	
WHO C	Negative control	59.28±3.58	59.08±2.03	54.90±7.30	51.66±4.94	
	Positive control	53.39±6.90	56.82±7.72	61.15±3.74	58.26±9.37	
	LT75	56.98±6.95	60.94±2.88	56.88±7.83	55.72±3.79	.394
	LT150	56.11±4.10	56.08±4.02	57.55±4.30	50.68±5.59	
	LT300	53.14±6.50	58.95±1.92	56.33±6.07	55.41±7.45	
WHO D	Negative control	15.26±4.08	15.48±3.56	14.03±6.149	22.13±10.25	
	Positive control	13.03±2.76	12.35±3.46	19.11±7.39	24.41±6.48	
	LT75	11.62±2.89	14.32±3.13	16.05±5.27	21.08±9.68	.791
	LT150	15.00±7.22	15.75±5.83	16.93±4.26	20.85±10.36	
	LT300	10.03±1.58	14.24±3.52	20.47±9.50	18.83±6.63	

Negative control= 0 (ovitamin C), Positive control 0+ (3000 µg/ml of vitamin C); LT75= ethanolic extract of Raphia hookeri added at dose 75 µg/ml; LT150=ethanolic extract of Raphia hookeri added at dose 150 µg/ml; LT300= ethanolic extract of Raphia hookeri added at dose 300 µg/ml; WHO A =Fast Progressive, WHO B =Slow Progressive, WHO C =Locale Motile WHO D =Immotile

Effects of ethanolic extract of *R. hookeri* fruits and the preservation period on fast progressive (WHO A), slow progressive (WHO B), locale motile (WHO C) and immotile (WHO D)

The results of the immotile spermatozoa showed no significant ($p > 0.05$) effect of the level of supplemented diluent amount the time of preservation. However, at 72 hour of preservation, the lowest percentage (18.83±6.63) was recorded at 300 µg/ml extract incorporation comparing to the other levels and the control groups (22.13±10.25% and 24.41±6.48%) respectively for the negative and positive control. The Effects of different doses of ethanolic extract of *Raphia hookeri* fruits and the preservation period on Fast Progressive (WHO A), Slow Progressive (WHO B), Locale Motile (WHO C) and the Immotile (WHO D) were presented in Table 5.

Table 6. Effects of different doses of ethanolic extract from *R. hookeri* fruits and the preservation period on average path velocity (VAP), Curvilinear Velocity (VCL) and Straight-line Velocity (VSL)

Characteristics	Treatments	Duration of storage				Interaction P-value
		0 H	24H	48H	72H	
VAP	Negative control	15.85±1.97	15.40±2.220	17.18±2.301	17.06±5.22	
	Positive control	17.73±2.35	19.39±6.62	14.71±3.76	13.59±5.58	
	LT75	16.94±2.17	16.33±2.20	16.43±3.89	15.74±4.14	.807
	LT150	16.81±3.37	16.44±2.42	16.89±3.35	15.89±4.812	
	LT300	17.15±2.86	16.13±1.03	18.05±6.585	16.57±4.96	
VCL	Negative control	25.17±3.29	24.48±3.72	27.86±3.74	28.97±8.66	
	Positive control	27.36±3.78	26.81±2.24	24.11±6.16	22.37±9.98	
	LT75	26.57±4.06	25.99±3.82	26.70±6.14	26.91±6.50	.949
	LT150	26.62±5.69	26.64±3.95	27.95±5.84	26.00±8.48	
	LT300	26.50±4.99	25.33±1.55	25.73±4.43	27.35±8.79	
VSL	Negative control	5.93±0.67	5.88±0.62	6.58±0.78	5.75±1.86	
	Positive control	6.87±1.03	6.60±1.17	5.43±1.16	5.05±1.72	
	LT75	6.63±0.98	5.93±0.63	5.97±1.37	5.53±1.71	.769
	LT150	6.30±1.22	6.04±0.93	5.95±0.96	5.94±1.80	
	LT300	6.97±0.56	6.11±0.47	5.54±1.34	5.89±1.50	

Negative control= 0 (ovitamin C), Positive control 0+ (3000 µg/ml of vitamin C); LT75= ethanolic extract of Raphia hookeri added at dose 75 µg/ml; LT150=ethanolic extract of Raphia hookeri added at dose 150 µg/ml; LT300= ethanolic extract of Raphia hookeri added at dose 300 µg/ml VAP =Average Path Velocity, VCL =Curvilinear Velocity; VSL =Straight-line Velocity

Table 7. Effects of different doses of ethanolic extract from raphia hookeri fruits and the preservation period on malodialdehyd (MDA), catalase (CAT), nitric oxide (NO), glutathione peroxidase(GPx) and superoxyde dismutase SOD

Characteristics	Treatments	Period de conservation		Interaction P-value
		0H	72H	
MDA (µM)	Negative Control	11.43±3.25	11.86±2.64	0.350
	Positive Control	9.74±1.64	9.90±2.22	
	LT75	11.32±2.01	12.99±0.91	
	LT150	12.83±2.04	12.36±0.77	
	LT300	8.53±1.76	11.60±2.30	
	Negative Control	47.92±8.08	34.55±8.89	0.171
(NO) (µM)	Positive Control	35.46±14.00	44.03±10.84	
	LT75	38.50±9.91	39.00±6.58	
	LT150	39.49±8.06	36.22±7.58	
	LT300	41.37±10.44	41.63±9.99	
	Negative Control	0.74±0.63	0.90±0.59	
	Positive Control	0.78±0.51	0.89±0.68	0.293
CAT (U/g Pt)	LT75	0.57±0.32	0.39±0.15	
	LT150	1.48±1.46	0.44±0.35	
	LT300	0.63±0.88	0.69±0.42	
	Negative Control	20.43±10.47	24.49±14.13	
	Positive Control	15.84±2.90	21.08±13.46	1.000
GPx (M/g Pt)	LT75	17.21±3.85	21.31±11.22	
	LT150	18.76±7.56	22.57±9.26	
	LT300	16.93±5.15	20.43±6.75	
	Negative Control	2.32±1.06	2.97±0.71	
	Positive Control	3.36±1.03	3.00±1.02	
	LT75	2.09±0.80	2.26±1.61	0.773
SOD (U/min/g Pt)	LT150	3.21±1.74	2.50±0.84	
	LT300	2.98±1.84	2.95±0.85	

SOD= Superoxyde dismutase GPx= Glutathione peroxidase; CAT= Catalase; MDA Malondialdéhyde NO= Nitric Oxide Negative control= 0 (ovitamin C), Positive control 0+ (3000 µg/ml of vitamin C); LT75= ethanolic extract of Raphia hookeri added at dose 75 µg/ml; LT150=ethanolic extract of Raphia hookeri added at dose 150 µg/ml; LT300= ethanolic extract of Raphia hookeri added at dose 300 µg/ml

Effects of ethanolic extract of *R. hookeri* fruits and the preservation period on average path velocity (VAP), curvilinear velocity (VCL) and straight-line velocity (VSL)

Statistically, no significant $p>0.05$ effect of interaction of time and various doses of supplementation in *Raphia hookeri* ethanolic extract was recorded. The lowest variability of the VAP during the period of preservation was noted in the sample supplemented at 150 µg/mL of extract comparing to the positive control where the lowest (22.37±9.98 µm/s) and the highest (26.81±2.24 µm/s) value of VAP were note down (Table 6).

Concerning the VCL, the value were very close in the different levels of supplementation and the neutral control but dissimilar to the positive control which had the highest variability.

Nevertheless, samples supplemented at 150µg/mL and stored at 72hours showed the highest (5.94±1.80 µm/s) VSL whereas at 0hour of storage, the highest 6.97±0.56 µm/s was register in the sample supplemented with 300µg/mL.

Effects of ethanolic extract of *R. hookeri* fruits and the preservation period on oxidative stress biomarkers malodialdehyd (MDA), catalase (CAT), nitric oxide (NO), glutathione peroxidase (GPx) and superoxyde dismutase SOD

No significant effect ($p>0.05$) has been recorded for the various doses of incorporation of ethanolic extract of *Raphia hookeri* on the different parameters (MDA, NO, SOD, CAT and GPx). However, at 300µg/mL of extract incorporation, the accumulation MDA was lower (8.53±1.76 and 11.60±2.30) respectively for 0 hour and 72 hour of preservation comparing to the negative control which accumulated (11.43±3.25 and 11.86±2.64) at the same period.

DISCUSSION

The important losses of semen quality during liquid storage limits the usage of stored semen to short time interval and hence represent a drawback for efficient application of assisted reproductive techniques such as artificial insemination (AI) and consequent improvement of production. The deleterious effects, which arise as a consequence of oxidative stress, on semen quality include decreases in motility, viability, membrane functionality, DNA integrity and enzymatic antioxidant activities, and the accumulation of toxic products such as Malondialdehyde (MDA). The results of the current study indicated that sperm quality parameters decline steadily throughout the storage period. However, *Raphia hookeri* at appropriate concentration and varying with the duration of storage can effectively reduce the decline of TM, PM, VCL, VSL, VAP, percentages of viable spermatozoa, spermatozoa with functional plasma membrane, and spermatozoa with normal morphology. The comparison of the results of sperm kinematics obtained from different experiments is challenging considering the variety of sperm concentrations in the samples and diluents used (Câmara *et al.*, 2011). A phytochemical analysis of *Raphia hookeri* extract showed the presence of phenols, flavonoids, tannins, anthocyanin and coumanin compounds which may provide a strong antioxidant activity. *Raphia hookeri* ethanolic extract could be used to inhibit MDA accumulation and stabilize SOD and CAT activities during low temperature liquid storage. Motility has been documented as one of the essential sperm parameters for fertility (Kasimanickam *et al.*, 2011), especially in AI procedures that require sperm cells to move within the reproductive tract of the females to reach the ovum. Effective semen storage relies on reversible decrease in motility and metabolic activity of sperm cells following cooling at lower temperatures; however, exposure of sperm cells to artificial conditions amplifies the generation of ROS which normally arises as a consequence of aerobic conditions where live sperm cells are involved (Agarwal *et al.*, 2010). As the ROS accumulate and reach a critical concentration, oxidative stress occurs and provokes an irreversible loss of motility, inhibition of fructolysis and respiration in sperm cells (Salamon and Maxwell, 2000), hence the decrease over time in sperm motility and motion characteristics as observed in the present study. Additionally, motility, which is an energy-dependent function, is particularly associated to mitochondrial activity and therefore may also decrease as a consequence of insufficient supply of energy from mitochondria which impairment drives to adenosine triphosphate (ATP) depletion. In fact, sperm

mitochondrion is particularly sensitive to cooling process and this sensitivity results in disturbance in ATP transport with consequent reduction in motility (Zarei *et al.*, 2021). Interestingly, all *Raphia hookeri* treated samples showed superior percentage of motile spermatozoa (POMS) in comparison to the control sample at 72 hour of preservation. Progressive motile sperm cells represent the spermatozoa fraction that can effectively move within the female reproductive tract once insemination is performed. Therefore, by reducing the loss of POMS, Raphia hookeri may improve the fertilization rate of chilled semen. Enrichment of skim milk extender with *Raphia hookeri* at 150µg/mL or 300µg/mL beneficially affected VSL for 72 hour of preservation. Moreover, the same supplementation is positively affected VCL and VAP from 0 to 48 hour of preservation. The preservation of these sperm attributes can be ascribed to the capacity of the bioactive components present in *Raphia hookeri* to inhibit the generation and/or scavenge ROS in excess. Particularly Raphia bioactive components may inhibit the mitochondrial outer membrane enzyme monoamine oxidase that catalyses the oxidative deamination of biogenic amines, producing a large amount of H_2O_2 that contributes to an increase in the steady state concentrations of reactive species within both the mitochondrial, matrix and cytosol (Cadenas and Davies, 2000). In this way, it may restore the balance between the amounts of ROS produced and scavenged, and consequently preserve the metabolic activity of sperm cells. It is well known that *Raphia hookeri* is a rich source of bioactive ingredients among which vitamin E which is considered as an essential component of the sperm antioxidant defence system, hence one of the major protector against oxidative stress and LPO (Ogbuagu, 2008). The presence in the Raphia fruits of vitamin E which is liposoluble, may have inhibited the peroxidation of PUFAs abundant in ram sperm membrane. Viability, as assessed by dye exclusion, allows discriminating the necrozoospermia from the total lack of motility associated to structural deficiencies in the tail zone (Chemes and Rawe, 2003). The results of this study evidenced the beneficial influence of all doses of incorporation especially from 24 to 72 hour on sperm viability during storage. Natural herbs cladodes (*Opuntia ficus indica*), green tea (*Camellia sinensis*) and Spirulina (*Arthrospira platensis*) used as additives to semen extenders improved viability (Allai *et al.*, 2016; Mehdipour *et al.,* 2016; Kameni *et al.*,2022). During semen storage, the accumulation of ROS above the detoxifying capacity of spermatozoa leads to peroxidative damage of membrane proteins, phospholipids, and PUFAs (Peris-Frau *et al.*, 2020; Kameni *et al.*, 2021), hence the loss of membrane integrity and subsequent cell death. This phenomenon is particularly prominent in ram because of the abundance of PUFAs in sperm plasma membrane (Bucak *et al.*, 2007). The improvement of sperm viability in the present study may be essentially linked to the bioactivity of anthocyanin which has been documented as the compound mainly responsible for the antioxidant activity due to its strong radical scavenging properties. Anthocyanin and other chemicals present in *Raphia hookeri* may have scavenged the ROS generated in excess, hence reducing ROS detrimental action on sperm membrane constituents, inhibiting LPO, and ultimately preserving sperm viability as noticed in this investigation. Furthermore, *Raphia hookeri* extract may have inhibited the release of cytochrome C from the mitochondria to the cytosol, release which is the initiation point of the apoptosis cascade (Silva, 2006). Besides improving the antioxidant defence of spermatozoa, *Raphia hookeri* may have strengthen the levels of phosphoinositide-3 kinases which have been documented as potent stimulators of several antiapoptotic effectors, hence preventing cell death (Oudit *et al.*, 2004). The results of the current work indicated that, *Raphia hookeri* at 75 µg/mL and 150µg/mL improved sperm functional membrane integrity at 72 hour. This observation may be associated to the capacity of *Raphia hookeri* extract to inhibit the generation of free radicals and their negative action on lipid bilayer interactions and proteins anchorage to the bilayer, ultimately preventing the loss of physiological function. In this study, as the storage was extended, the percentages of sperm morphological abnormalities increased. This result is in accordance with previous reports (Gundogan *et al.*, 2011; Gheller *et al.*, 2018). However, enrichment of extenders with *Raphia hookeri* extract at highest concentration (300µg/mL) showed to be effective at reducing sperm morphological defects at

all the various period of preservation. For satisfactory results following AI in small ruminants, the threshold of 15% has been suggested as the maximum critical percentage of sperm morphological defects (Rehman *et al.*, 2013). While many studies have reported no effect following extender supplementation with antioxidant compounds on sperm morphology (Amini *et al.*, 2019; Zarei *et al.*, 2021), arguing that morphology is mainly related to spermatogenesis, others have highlighted positive effects (Allai *et al.*, 2016; Rateb, 2018). It seems as the dynamics of sperm morphology expands beyond the scope of spermatogenesis, and morphology alteration might also be prevented due to the antioxidative potential of *Raphia hookeri* extract. Moreover, the nature, chemical composition and incorporation level of the antioxidant compound coupled with the variety of methodologies used to assess sperm morphology may account for the discrepancy observed. Recent investigations have evidenced the increase of the antioxidant capacity of semen and the inhibition of LPO following addition of antioxidants from natural origin to extenders during semen storage (Wen *et al.*, 2019, Taşdemir *et al.*, 2020). Approximately, the enrichment of skim milk extender with *Raphia hookeri* stabilize SOD and CAT levels and inhibited MDA accumulation during cooling storage at 5°C. Under low LPO rates (sub-toxic conditions), cells initiate their maintenance and survival through intrinsic antioxidant defence systems or signalling pathways activation that up-regulate protein antioxidants resulting in an adaptive stress response. On the other hand, under medium or high LPO rates (toxic conditions), the magnitude of oxidative stress exceeds repair capacity, and the cells induce apoptosis or necrosis (Ayala *et al.*, 2014). Therefore, by moderating the rate of LPO as observed in the present work, *Raphia hookeri* may have created subtoxic conditions that favour the increase of enzymatic antioxidant activities and the preservation of sperm quality.

CONCLUSION

This study demonstrated that the ethanolic extract of *Raphia hookeri*, had permeated to maintain a relative stability in motility, viability, plasma membrane integrity, and DNA integrity, even after several days of storage. These results suggest that *Raphia hookeri* extract may act as a protective agent, limiting the formation of reactive oxygen species and thereby preserving sperm function. Furthermore, this plant offers a natural, economical, and accessible alternative for improving sperm quality in the African context, where resources for sperm storage are limited. In summary, the use of *Raphia hookeri* extract in preservation diluents could help improve the effectiveness of artificial insemination by increasing the post-preservation success rate and supporting the development of the sheep industry in the region.

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